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A TRYPSIN INHIBITOR FROM THE COELOMIC FLUID OF THE SEA STAR *ASTERIAS FORBESI*

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Abstract

Cell-free coelomic fluid obtained from the sea star *Asterias forbesi* rapidly neutralized, in a dose-dependent fashion, trypsin derived from several different animal species. Approximately 50 to 300 μg of bovine trypsin was inhibited per mg of total sea star coelomic fluid protein. Crude preparations of sea star and sea urchin trypsins isolated from gastric tissue, as well as purified human plasmin, were also neutralized upon addition to echinoderm coelomic fluid. In contrast, bovine α_1-chymotrypsin and thrombin were not inactivated by the coelomic fluid inhibitor. The trypsin inhibitor was only mildly sensitive to heat or acid treatments and exhibited a molecular weight of ~6500. The protease inactivator was also detected in cell lysates derived from washed coelomocytes collected in N-ethyl maleimide, as well as in cell-free coelomic fluid collected by non-surgical means.

Introduction

Trypsin represents a constitutive protease of most organisms, including bacteria (Winter and Neurath, 1970; Okabe and Noma, 1974; Russo and Yadoff, 1978; Goldberg *et al.*, 1980; Zwilling and Neurath, 1980). Trypsin inhibitors also have been detected within a wide variety of organisms, including invertebrates (Greene *et al.*, 1976; Tschesche, 1976; Tschesche and Dietl, 1976; Holzman and Russo, 1978). The invertebrate trypsin inhibitors inactivate sundry enzymes, including chymotrypsin. In the present communication, the detection and partial characterization of a sea star coelomic fluid inhibitor, which inactivates trypsin but not chymotrypsin, is described.

Materials and Methods

All chemicals and reagents were purchased from either Sigma Chemical Co. (St. Louis, Missouri) or Fisher Scientific (Pittsburgh, Pennsylvania) and were reagent quality or better. Synthetic substrates were obtained from Helena Laboratories (Beaumont, Texas). Bovine thrombin was purchased from Parke-Davis (Morris Plains, New Jersey). Sea stars (*Asterias forbesi*) weighing ~150 gm were obtained daily from the Supply Department of the Marine Biological Laboratory (Woods Hole, Massachusetts) and used only once.

Coelomic fluid was obtained by surgically amputating the tips of sea stars' arms and draining the fluid into chilled, pyrogen-free glass tubes. After centrifugation at 100 × *g* for 5 min at 4°C, the supernate was decanted and represented cell-free coe-

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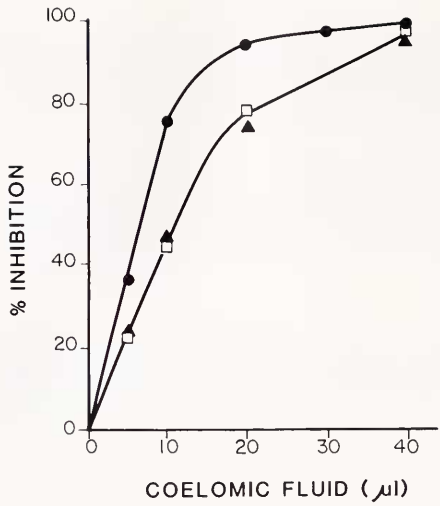

FIGURE 1. Inhibition of bovine trypsin by sea star cell-free coelomic fluid. Increasing amounts of coelomic fluid were pre-incubated with 0.1 μg of bovine trypsin for 10 min at 22°C (410 μl, final volume). Residual proteolytic (- ● -) activity was quantitated with the chromogenic substrate S2222 as described in Materials and Methods. Similar studies were conducted with coelomic fluid after heating to 100°C for 5 min (- □ -) and after acidification to pH 2.5 for 1 h at 24°C and neutralization (- ▲ -).

lomic fluid. The pellet, consisting of coelomocytes, was suspended in pyrogen-free, distilled water (one ml per animal). The cellular pellet was disrupted by frequent and vigorous shaking for 2 h at 22°C. Cellular debris was removed by centrifugation at 1000 × *g* for 10 min at 22°C. The supernate was decanted and represented the cell lysate. Phase contrast microscopy revealed that >90% of the cells were lysed under these conditions.

To measure the inhibitor activity of sea star cell-free coelomic fluid or cell lysate, residual enzymatic activity was quantitated employing chromogenic substrates. The assay was initiated by mixing coelomic fluid or cell lysate (5–400 μl) with 10 μl of enzyme for 10 min at 22°C (410 μl total volume). Controls were constructed by substituting the environmental buffer, 0.45 *M* NaCl in 50 m*M* Tris-HCl, pH 7.5, for the test sample. Chromogenic substrate (100 μl) was added to the above solution at a final concentration of 0.5 m*M*, and the reaction mixture was incubated for 5 min at 22°C and quenched with 200 μl of glacial acetic acid. Amidolysis was quantitated by reading the absorbance at 405 nm. The fraction of inactivated enzymatic activity was calculated by dividing residual amidolytic activity in either coelomic fluid or cell lysate by total enzymatic activity determined in the absence of these samples.

Protein concentrations were quantitated by the procedure of Bradford (1976) using bovine serum albumin as standard (Sigma, fraction V).

RESULTS AND DISCUSSION

Addition of bovine trypsin (0.1 μg) to cell-free coelomic fluid resulted in rapid neutralization of the enzyme in a dose-dependent fashion (Fig. 1). As little as 5 μl of the sea star coelomic fluid inhibited ~40% of the added protease. The specific activity of the inhibitor under these conditions ranged from 50 to 300 μg of trypsin inhibited per mg of total coelomic fluid protein (8 animals were tested). As shown in Figure 1,

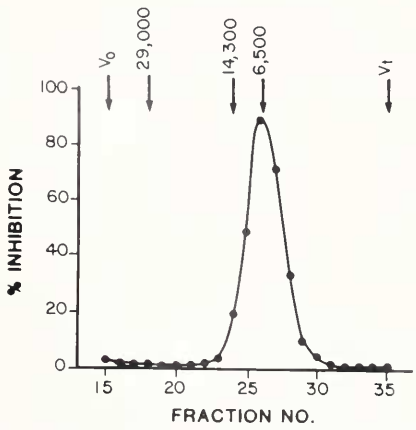

FIGURE 2. Gel permeation of sea star cell-free coelomic fluid. Coelomic fluid (200 μl) was gel filtered on Sephadex G-50 (0.6 cm $\times$ 115 cm) equilibrated in 0.45 *M* NaCl in 50 m*M* Tris-HCl, pH 7.5. Fractions of 0.9 ml were collected at a column flow rate of 9 ml/h. Inhibition of bovine trypsin by eluate fractions was determined by measuring residual enzymatic activity with S2222 as outlined above. Molecular weight standards included carbonic anhydrase (29,000), lyzozyme (14,300), and aprotinin (6500).

the inhibitor is only mildly sensitive to heat and acid treatments. A molecular weight of about 6500 was estimated by gel permeation chromatography using Sephadex G-50 (Fig. 2). The above molecular characteristics of the coelomic fluid inhibitor are similar to those described for other small molecular weight inhibitors (Fritz and Krejci, 1976; Tschesche, 1976; Tschesche and Dietl, 1976).

Crude preparations of proteolytic enzymes were obtained from the gastric tissues of *A. forbesi* and the sea urchin *Strongylocentrotus droebachiensis*. Briefly, the gut was excised surgically and disrupted with a glass pestle and homogenizer (Radnoti Glass Technology, Inc., Monrovia, California 91016). Cellular debris was removed by centrifugation at 1000 $\times$ *g* for 20 min at 4°C, and the supernate containing the gut enzyme(s) was decanted. These gut proteases represented trypsin-like enzymes based upon the following criteria: (1) Enzymatic activity was inhibited completely by bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor, and (2) the proteases hydrolyzed synthetic peptide substrates containing carboxy-terminal arginyl residues (data not shown). Admixture of protease from either sea star or sea urchin with sea star coelomic fluid resulted in rapid inhibition of the respective enzymatic activity (Table I). Inhibition of these trypsin-like enzymes was dose-dependent, with ~40% of the enzymatic activity being inactivated by 5 μl of coelomic fluid (data not shown). Plasminogen was purified from human plasma (Deutsch and Mertz, 1970) and activated with streptokinase (Marcum *et al.*, 1982). Plasmin was rapidly neutralized upon addition to sea star coelomic fluid (Table I).

In contrast, bovine α_1-chymotrypsin was not neutralized by the sea star coelomic fluid inhibitor (Table I). As little as 10 ng of the protease was not inactivated upon addition to sea star coelomic fluid (200 μl). The above data indicates that the specificity of the protease inhibitor is distinct from the Kunitz inhibitor which neutralizes trypsin and chymotrypsin (Kunitz and Northrop, 1936), but similar to the Kazal inhibitor which inactivates trypsin but not chymotrypsin (Kazal *et al.*, 1948). The majority of invertebrate inhibitors described to date are Kunitz-type inhibitors, whereas only the bdellins isolated from leeches are Kazal-type inhibitors (Fritz and

TABLE I

Inactivation of enzymes by coelomic fluid inhibitor

Enzyme[1]	% Inhibition
Bovine trypsin	100
Sea star trypsin	100
Sea urchin trypsin	100
Human plasmin	100
Bovine α_1-chymotrypsin	0
Bovine thrombin	0

[1] Residual proteolytic activity was quantitated utilizing chromogenic substrate S2222 for measuring 0.1 μg bovine pancreatic trypsin (12,400 BAEE units/mg), S2238 for 0.1 unit bovine thrombin (56 NIH units/mg), S2586 for 0.01 to 1 μg bovine pancreatic chymotrypsin (50 BTEE units/mg), S2444 for 5.6 μg sea star gut-extracted protease(s) (197 BAEE units/mg), and S2238 for 3.3 μg sea urchin gut-extracted protease(s) (149 BAEE units/mg). In the case of human plasmin (1 μg), enzymatic activity was measured employing ^{125}I-labeled α-casein as substrate (Highsmith and Rosenberg, 1977).

Krejci, 1976). It must be noted, however, that Kazal inhibitors inactivate thrombin, whereas the sea star inhibitor did not neutralize a crude preparation of bovine thrombin (Table I).

To determine whether the inhibitor is present in coelomocytes, coelomic fluid was collected in N-ethyl malemide (5 m*M*, final concentration), a potent stabilizer of plasma membranes (Bryan *et al.*, 1964). Analysis of washed coelomocytes collected under the above conditions revealed that lysate preparations of these cells also contained the trypsin inhibitor. However, the specific activity of lysates obtained from washed coelomocytes (5.89 μg trypsin inhibited/mg total protein) was ~20-fold less than the specific activity of the cell-free coelomic fluid (90 μg trypsin inhibited/mg total protein). These results suggest that the trypsin inhibitor is a natural component of coelomocytes and possibly synthesized by these cells and secreted into the coelomic fluid.

Experiments conducted with cell-free coelomic fluid, which was collected by mechanical manipulation without damage to sea stars, revealed that trypsin inhibitor levels within the above fluid were comparable to amounts quantitated for coelomic fluid obtained by surgical means. Bovine trypsin was inhibited in a dose-dependent fashion, with ~40% of the protease inactivated by 5 μl of the coelomic fluid (data not shown). The specific inhibitor activity of coelomic fluid collected by non-surgical methods (250 μg trypsin inhibited/mg total protein) was comparable to those obtained by surgical removal of the tips of the sea star arm (200 μg trypsin inhibited/mg total protein).

In conclusion, an inhibitor which inactivates trypsin but not chymotrypsin has been detected in the coelomic fluid of the sea star *Asterias forbesi*. The above data suggest that the trypsin inhibitor within the coelomic fluid of this invertebrate resembles the Kazal-type inhibitors of vertebrates. Although the physiological function of the protease inhibitor remains unclear, it may involve scavenger activity in neutralizing trypsin, which appears in the coelomic fluid, or host defense mechanisms.

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